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RESEARCH ARTICLE

# Copper-induced liver fibrosis affects the behavior of bone marrow cells in primary culture

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**BACKGROUND:** The present study investigated the effects of low-molecular-weight components of bovine colostrum (LMCC), which were administered *per os*, on the differentiation, growth, and survival of cells obtained from the bone marrow of rats in primary culture.
**METHODS:** Bone marrow cells (BMCs) were obtained from both the rat femurs and were cultured in medium 199 supplemented with antibiotics (8% streptomycin and 8% gentamycin) and 20% inactivated fetal calf serum. In addition, the number of BMCs was counted and their morphotypes were determined.
**RESULTS:** Animals were treated with copper (Cu) sulfate to induce liver fibrosis. Subsequent treatment with LMCC eliminated growth inhibition and normalized the bodyweight and temperature of animals with Cu-induced liver fibrosis. The number of lymphocytes in the bone marrow of animals with Cu-induced liver fibrosis was significantly higher than that in the bone marrow of control animals. The number of neutrophils in untreated animals with liver fibrosis and LMCC-treated animals with liver fibrosis was lower than that in control animals. Neutrophils obtained from untreated animals with liver fibrosis and LMCC-treated animals with liver fibrosis underwent two-times faster degradation *in vitro* than neutrophils obtained from control animals.
**CONCLUSIONS:** Our results indicate that LMCC affects the distribution of different morphological types of BMCs but does not prevent their degradation *in vitro*, which was two-times faster than that of BMCs obtained from control animals.

**Keywords** bone marrow cells, bone marrow, colostrum, liver fibrosis

## Introduction

The bone marrow is a unique dynamic system containing many different cell and tissue types. It produces red blood and immune cells, maintains the regenerative capacity of the body, and is a key depot of stem cells (Al-Nbaheen et al., 2013; Morrison et al., 2013; Morrison and Scadden, 2014).

Proliferation and differentiation of bone marrow cells (BMCs) are strictly regulated by the structured microenvironment of the bone marrow and by various signaling pathways involving interleukins, interferons, various growth factors, and cell–cell interactions (Krause et al., 2013).

Toxic compounds, physical factors, and radiation affect the microenvironment of the bone marrow and alter signal transduction pathways that may change the proliferation and differentiation rates of BMCs (Greim et al., 2014; Yagi et al., 2013), leading to the development of different pathologies (Kidd et al., 2012; Nwajei and Konopleva, 2013; Schepers et al., 2013).

To understand mechanisms underlying the regulation and differentiation of BMCs, it is necessary to develop new approaches for evaluating their behavior in response to exogenous stimuli.

Exogenous toxic compounds and different pathologies arising in other tissues affect the bone marrow microenvironment (Spahr et al., 2013; Terai et al., 2014).

Mechanisms underlying the effect of the bone marrow microenvironment on the behavior of BMCs can be determined by studying the integrative functions of the bone marrow. This can be successfully performed by developing an experimental model of liver fibrosis.

Sequential administration of copper (Cu) sulfate in experimental animals under oxidative stress results in the development of liver fibrosis (Bozhkov et al., 2014).

Liver fibrosis affects bone marrow function. Mesenchymal

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stem cells in the bone marrow move to inflammatory foci to stimulate liver regeneration (Sánchez-Soto et al., 2017).

It is interesting to investigate the in vitro proliferation ability of BMCs obtained from control animals and animals with liver fibrosis and to determine the lifespan of these cells in primary culture. Results of these analyses will help in assessing the possible effects of liver fibrosis on the characteristics of BMCs.

Cu-induced liver fibrosis alters cytokine profile, prooxidant–antioxidant system, and indicators of redox system(Sánchez-Valle et al., 2014).

We believe that changes in the characteristics of the redox system will affect both BMC function and fibrosis development.

Bovine colostrum contains different biologically active compounds (Godhia1 et al., 2013; Rathe et al., 2014; Bagwe et al., 2015; Conneely et al., 2014; Blach-Olszewska et al., 2015) and is used for treating Alzheimer's disease, multiple sclerosis, Crohn's disease, rheumatoid arthritis, and other diseases (Folch et al., 2016; Kochs et al., 2014; Uto et al., 2015; Terato et al., 2015).

Moreover, bovine colostrum contains heavy immunoglobulins that induce allergic reactions (Conneely et al., 2013).

Oxidative stress, which is triggered by the accumulation of exogenous Cu ions in the liver, acts as a systemic inducer of adaptive reactions in the body. This results in the rapid restructurization of the redox system of the whole organism, including the bone and brain. Alteration of the bone marrow microenvironment is accompanied by changes in cell proliferation and differentiation rates, which contribute to the restoration of oppressed liver functions.

Low-molecular-weight components of bovine colostrum (LMCC) lacking fat and high-molecular-weight proteins are suggested to affect the development of liver fibrosis. .

LMCC has antioxidant and immunotropic effects (Mathias et al., 2014) and can be used for treating liver fibrosis (Conneely et al., 2014). It is interesting to determine the effect of LMCC on BMCs obtained from control animals and animals with liver fibrosis.

Results of this analysis will help determine whether LMCC alters the proliferation and lifespan of BMCs obtained from animals with liver fibrosis.

In the present study, we determined the effect of bovine LMCC (containing proteins with molecular weights of$\leqslant$45 kDa) on the distribution pattern of main BMC types (morphotypes) and lifespan of lymphocytes and neutrophils obtained from control animals and animals with liver fibrosis in primary culture.

## Materials and methods

### Experimental scheme for establishing study animals

Experiments were performed using 3-month-old, male Wistar rats. All animal experiments were performed in compliance with bioethical principles (Council Directive 86/609/EEC,1986) by considering the circadian rhythms of biological responses. For this, the animals were always given food at the same time and maintained under standard conditions and all experiments (weight and temperature measurement and toxic or other drug administration) were performed at the same time before feeding. The animals were fasted for 24 h before isolating BMCs, and BMC isolation was always performed from 8 to 10 AM.

The animals were divided into three groups of 8 animals each: (1) control animals, (2) animals with Cu-induced liver fibrosis, and (3) animals with Cu-induced liver fibrosis treated with LMCC. The experimental scheme for establishing the study animals is shown in Fig. 1.

### Measurement of somatometric indicators in experimental animals

Bodyweight of the animals was measured on days 1, 3, 7, 11, 13, 15, and 19 of the experiment. The graphs of change in bodyweight compared with the initial bodyweight were plotted.

Rectal temperature of the animals was measured on days 1, 3, 7, 11, 13, 15, and 19 of the experiment, using a thermometer (MicroTherma 2T Hand Held; Braintree Scientific, Inc., USA).

### *Isolation and cultivation of BMCs* in vitro

BMCs were obtained from both the femurs of animals in all the experimental groups, according to a method described previously (Javazon et al., 2004), and cultured in medium 199 supplemented with antibiotics (8% streptomycin and 8% gentamicin) and 20% inactivated fetal calf serum. The BMCs were cultivated for 4 days under standard conditions at 37°C and in an atmosphere of 5% $CO_2$. The culture medium was not removed, and the initial concentration of the BMCs in the culture was maintained at 2 million cells/ml.

### Determination of different morphological types of BMCs and their number

The number and viability of BMCs were determined, as described previously (Bozhkov et al., 2014). BMC morphotypes were determined immediately after isolating the cells from the bone marrow and on days 2 and 4 of cultivation, as described previously (Bozhkov et al., 2014). The cells were stained with Romanowsky–Hymsa stain and were analyzed at 100 $\times$ magnification using Primo Star iLED microscope (Zeiss, Germany).

### Statistical analysis

Mean, standard deviation, standard error of the mean, and

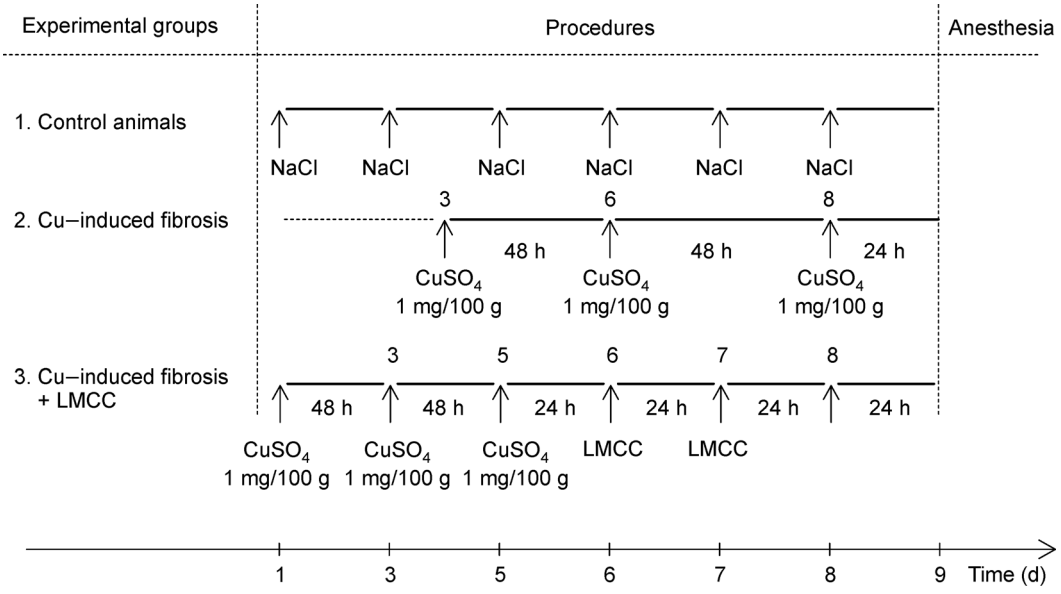


**Figure 1** Scheme showing the sequential administration of Cu sulfate at a dose of 1 mg/100 g liver weight, with 48-h intervals between each administration for inducing liver fibrosis (group 2).Triple LMCC administration *per os* was performed at 24-h intervals between each administration (group 3).Control animals were injected with saline instead of Cu sulfate (group 1). BMCs were obtained by anesthetizing the animals in all the experimental groups with ether

sample volume were used as sample characteristics. Significant differences between groups were determined by a nonparametric Mann–Whitney *U* test. All statistical analyses were performed using MS Excel program. Differences between control and experimental groups were considered significant at $p \leqslant 0.05$.

## Results

### Somatometric indicators in experimental animals

Sequential triple administration of Cu sulfate in experimental animals at a dose of 1 mg/100 g bodyweight, which amounted to 33% of fatal dose, induced liver fibrosis (Bozhkov et al., 2010) and bodyweight loss, which was pronounced at the beginning of the experiment. Cessation of Cu sulfate administration in experimental animals gradually restored the bodyweight, with the bodyweight on day 13 of the experiment being the same as that of control animals (Fig. 2A).

Loss of bodyweight in these animals was associated with Cu ion-induced inhibition of metabolism, which significantly decreased the body temperature of these animals compared with that of control animals (Fig. 2B).

Animals with liver fibrosis treated with LMCC (0.1 mg/100 g bodyweight) did not show a decrease in bodyweight and showed rapid recovery of slight growth retardation; moreover, the bodyweight of these animals was not different from that of control animals (Fig. 2A). Furthermore, the body temperature of these animals did not significantly differ from that of control animals (Fig. 2B).

Administration of a low dose of LMCC (0.05 g/100 g bodyweight) in animals with Cu-induced liver fibrosis exerted a similar effect on bodyweight; however, this effect was less pronounced than that exerted by the LMCC dose of 0.1 mg/100 g bodyweight (Fig. 2A).

These results indicate that LMCC administration in animals with Cu-induced liver fibrosis reversed the changes in somatometric indicators specific to liver fibrosis and that the effects of the LMCC dose of 0.1 g/100 g bodyweight were more pronounced than those of the LMCC dose of 0.05 g/100 g bodyweight.

Therefore, we used the LMCC dose of 0.1 g/100 g bodyweight in all subsequent experiments.

### Changes of the number of BMCs in primary culture

Total number of BMCs obtained from control animals increased almost linearly from days 1 to 4 of cultivation (Fig. 3). It should be noted that on the medium 199 supplemented with 20% serum, the number of BMCs for 96 h of cultivation increased by 2.0-2.5 times.

BMCs obtained from animals with liver fibrosis showed higher proliferation rate during the first 24 h of cultivation than BMCs obtained from control animals. However, their number decreased and remained constant over 96 h of cultivation, with the number of BMCs obtained from animals with liver fibrosis being 2-fold lower than that of BMCs obtained from control animals after 96 h of cultivation (Fig. 3).

In contrast, BMCs obtained from animals with liver

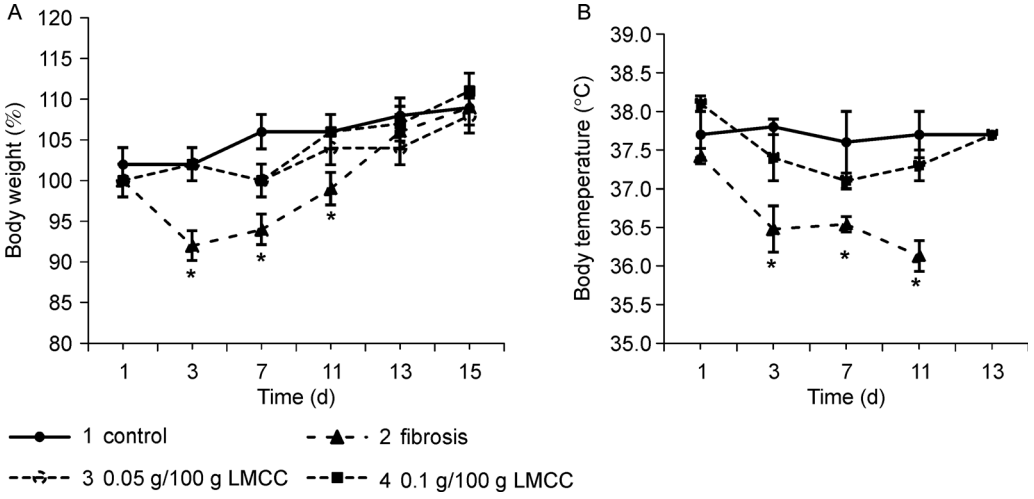


**Figure 2** Bodyweight and body temperature of control animals (1), animals with Cu-induced fibrosis (2), and animals with Cu-induced fibrosis treated with LMCC at a dose 0.05 g/100 g bodyweight (3) or 0.1 g/100 g bodyweight (4) (A).Changes in the rectal temperature of control animals (1), animals with Cu-induced fibrosis (2), and animals with fibrosis treated with LMCC at a dose 0.1 g/100 g bodyweight (B). * $p \leqslant 0.05$ compared with the control animals.

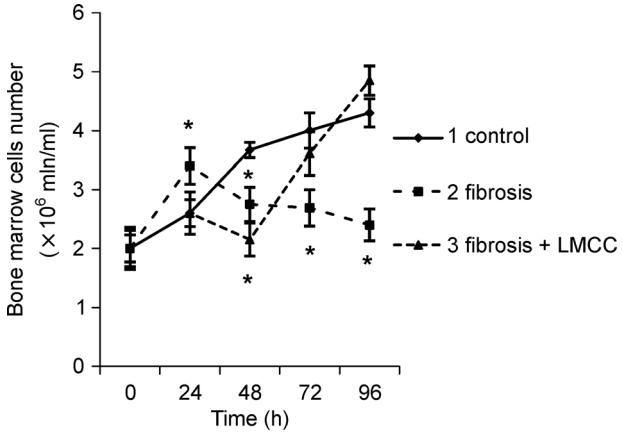


**Figure 3** Number of BMCs in primary culture from days 1 to 4 of cultivation in medium 199 supplemented with 20% fetal calf serum. BMCs were obtained from control animals (1), animals with Cu-induced liver fibrosis (2), and animals with Cu-induced fibrosis treated with a triple dose of LMCC administered at 24-h intervals (3). * $p \leqslant 0.05$ compared with the control animals.

fibrosis treated with LMCC showed different growth dynamics (Fig. 3).

Thus, in the first 24 h of cultivation, the number BMCs obtained from animals with liver fibrosis treated with LMCC was not different from that of BMCs obtained from control animals. After 48 h of cultivation, the number of BMCs obtained from animals with liver fibrosis treated with LMCC was lower than that of BMCs obtained from control animals and did not differ from that of BMCs obtained from animals with fibrosis not treated with LMCC. After 96 h of cultivation, the number of BMCs obtained from animals with liver fibrosis treated with LMCC increased sharply (by 2.5 fold) and did not differ significantly from that of BMCs obtained from control animals (Fig. 3).

Thus, BMCs obtained from animals in the three experimental groups showed different growth dynamics in primary culture, i.e., slow growth for BMCs obtained from control animals; fast growth and subsequent degradation for BMCs obtained from animals with liver fibrosis; and slow growth for 48 h, followed by rapid growth from 48 to 96 h for BMCs obtained from animals with liver fibrosis treated with LMCC (Fig. 3).

These differences in the growth dynamics of cultured BMCs obtained from animals in the different experimental groups may be because of the active response of BMCs to different metabolic states induced by liver fibrosis and LMCC administration. These metabolic states were accompanied by changes in the proliferation rate of BMCs in primary culture.

The distribution pattern of morphotypes of BMCs depends on the bone marrow microenvironment. Characterization of the distribution pattern of different cell morphotypes in the bone marrow is important for determining bone marrow activity.

To clarify this, we determined the number of different cell types (morphotypes) in the bone marrow.

## Number of different cell morphotypes in the bone marrow of animals in different experimental groups

The bone marrow of control animals contained approximately 18% stab neutrophils; 10% metamyelocytes; approximately 8% lymphocytes; > 6% segmented neutrophils; almost the same number of myelocytes and eosinophils; and 0.65% and 0.55% basophils and monocytes, respectively (Fig. 4). Thus, approximately 53% cells could be morphologically identified in the bone marrow of control animals. Mesenchymal stem cells and undifferentiated cells accounted for the remaining 47% cells in the bone marrow of control animals.

The ratio of different morphological cell types in the bone

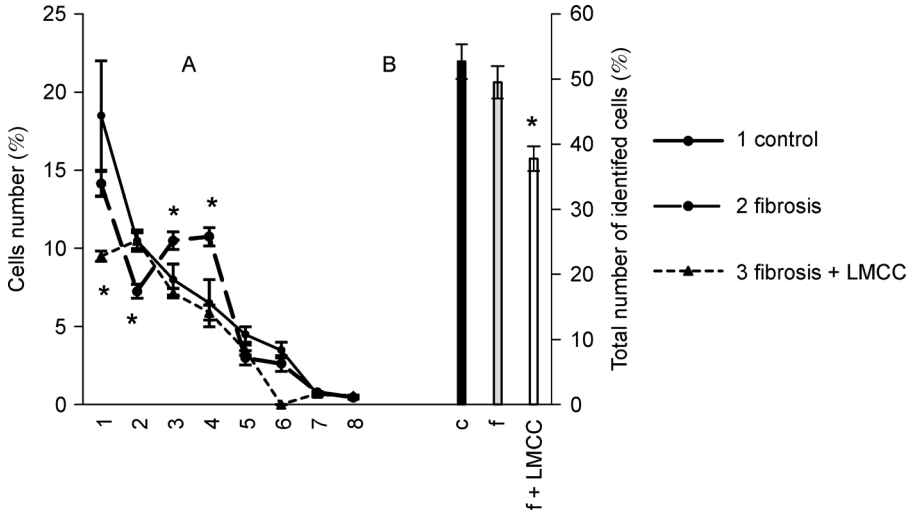


**Figure 4** Number of different BMC morphotypes cell suspension (A) and total number of identified cells (B) in control animals, animals with liver fibrosis, and animals with fibrosis treated with a triple dose of LMCC (0.1 g/100 g bodyweight *per os*) administered at 24-h intervals. On axis: 1, stab neutrophils; 2, metamyelocytes; 3, lymphocytes; 4, segmented neutrophils; 5, myelocytes; 6, eosinophils; 7, basophils; and 8, monocytes. * $p \leqslant 0.05$ compared with the control animals.

marrow of animals with Cu-induced liver fibrosis was different from that in the bone marrow of control animals (Fig. 4). The bone marrow of animals with fibrosis contained approximately 14% stab neutrophils; 7% metamyelocytes; approximately 11% lymphocytes; > 10% segmented neutrophils; almost the same number of myelocytes and eosinophils; and 0.79% and 0.46% basophils and monocytes, respectively (Fig. 4).

The distribution pattern of different morphotypes in the bone marrow of animals with liver fibrosis treated with LMCC was different from that in the bone marrow of animals with liver fibrosis and control animals (Fig. 4).

The total percentage of BMC morphotypes was different in the bone marrow of animals in the different experimental groups, with 49% BMC morphotypes being present in the bone marrow of animals with fibrosis and only 35% BMC morphotypes being present in the bone marrow of animals with fibrosis treated with LMCC, which was significantly lower than that in the bone marrow of untreated animals with liver fibrosis and control animals (Fig. 4).

LMCC administration decreased the number of differentiated cell types but maintained the total number of cells in the bone marrow of animals with fibrosis. Moreover, LMCC administration changed the ratio of different cell morphotypes in the bone marrow of animals with fibrosis, suggesting an increase in the transport rate of BMCs into the blood and/or an increase in the degradation rate of these cell types.

In subsequent experiments, we determined the lifespan of BMCs in primary culture.

### Lifespan of lymphocytes in *in vitro* culture

Lymphocytes are immunocompetent cells produced from stem cells present in the bone marrow (Sprent and Tough, 1994). Naïve lymphocytes present in the bone marrow give rise to different specialized cells, including T cells (suppressor, killer, effector, helper, and memory cells) and B cells (helper, suppressor, and killer cells) (Ratajczak et al., 2004). The lifespan of lymphocytes in the blood varies considerably from several days to years (Alaribe et al., 2013). The number of lymphocytes obtained from control animals remained constant during 96 h of cultivation (Fig. 5).

The number of lymphocytes was significantly higher in the bone marrow of animals with Cu-induced liver fibrosis than in the bone marrow of control animals. Moreover, this high number of lymphocytes obtained from animals with liver fibrosis was maintained throughout the cultivation period (Fig. 5).

If the number of lymphocytes in the bone marrow of animals with fibrosis was 31% higher than that in the bone marrow of control animals, this number increased by 90% after 96 h of cultivation (Fig. 5). These results indicate that the ratio of different cell types obtained from the bone marrow of animals in the different experimental groups varied in primary culture and the number of cells obtained from the bone marrow of animals with fibrosis increased during cultivation.

Moreover, these results suggest that the behavior of lymphocytes obtained from control animals and animals with Cu-induced liver fibrosis was different. These differences in the behavior of lymphocytes in primary culture may be associated with epigenetic changes in BMCs after the development of liver fibrosis or may be because the bone marrow of animals with liver fibrosis contains more number of naïve lymphocytes than mature lymphocytes.

LMCC administration in animals with liver fibrosis did not change the number of naïve lymphocytes in the bone marrow and the behavior of these lymphocytes compared with that of lymphocytes in the bone marrow of control animals (Fig. 5).

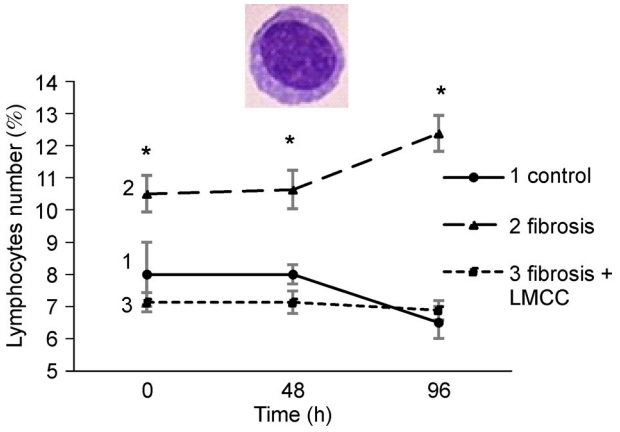


**Figure 5** Number of lymphocytes obtained from the bone marrow of control animals (1), animals with Cu-induced liver fibrosis (2), and animals with Cu-induced fibrosis treated with a triple dose of LMCC administered at 24-h intervals (3) from days 1 to 4 of cultivation. * $p \leqslant 0.05$ compared with the control animals.

Thus, LMCC administration may potentially inhibit fibrosis-induced immune response, as indicated by the normalization of lymphocyte number, or protect against the harmful effects of toxicants in animals with liver fibrosis.

## Lifespan of neutrophils in *in vitro* culture

Neutrophils are the most common group of leucocytes. Neutrophil maturation from stem cells occurs in the bone marrow and involves the following stages: progranulocytes → medulla cell → metamyelocytes → stab neutrophils. The half-life of neutrophils in the blood ranges from 6 h to 2–3 days (Kolaczkowska and Kubes, 2013).

The number of myelocytes in the bone marrow of animals with liver fibrosis was 34% lower than that in the bone marrow of control animals. LMCC administration in these animals did not affect myelocyte number compared with that in untreated animals with fibrosis (Fig. 6A).

The number of myelocytes obtained from the bone marrow of control animals remained unchanged after 48 h of cultivation and decreased by only 45% compared with their initial number after 96 h of cultivation (Fig. 6). The number of myelocytes obtained from the bone marrow of animals with fibrosis was 67% lower than their initial number after 48 h of cultivation. Moreover, these cells were completely eliminated after 96 h of cultivation (Fig. 6A). These results indicate that the lifespan of myelocytes from the bone marrow of animals with fibrosis was shorter than that of myelocytes from the bone marrow of control animals.

LMCC administration in animals with liver fibrosis did not affect the elimination rate of myelocytes in culture compared with that of myelocytes obtained from untreated animals with fibrosis (Fig. 6A).

This suggests that myelocytes obtained from animals with fibrosis undergo epigenetic changes at an early stage of stem cell differentiation that prevent their survival in culture compared with that of myelocytes obtained from control animals. These results indicate that LMCC administration does not affect the lifespan of myelocytes in primary culture.

The next stage of leukocyte differentiation in the bone marrow is the formation of metamyelocytes. The number of metamyelocytes obtained from control animals remained constant compared with their initial number after 48 h of cultivation and decreased by 24% compared with the initial number after 96 h of cultivation, indicating that the growth dynamics of metamyelocytes were similar to those of myelocytes (Fig. 6B). The rate of decrease in the number of metamyelocytes obtained from animals with liver fibrosis was

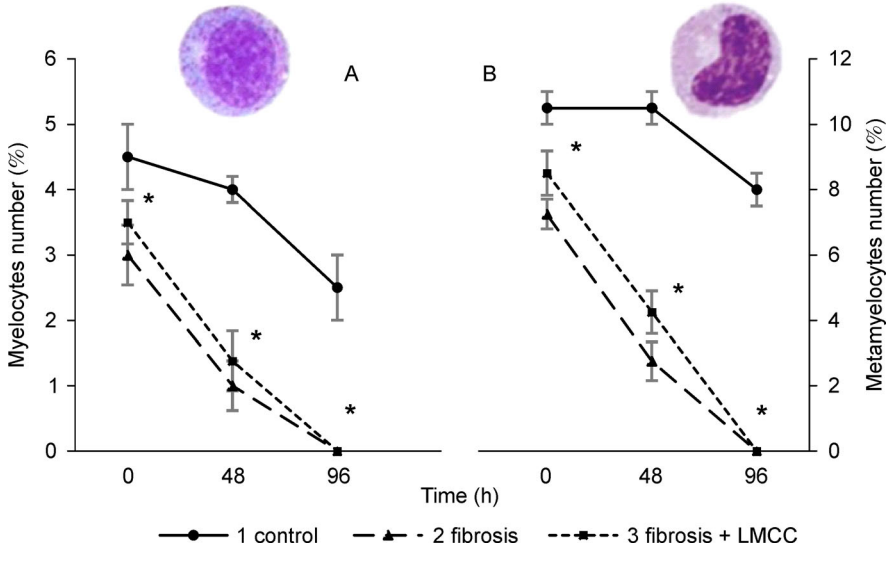


**Figure 6** Numbers of myelocytes (A) and metamyelocytes (B) obtained from the bone marrow of control animals (1), animals with Cu-induced liver fibrosis (2), and animals with Cu-induced fibrosis treated with a triple dose of LMCC administered at 24-h intervals (3) during *in vitro* cultivation. * $p \leqslant 0.05$ compared with the control animals.

linear in primary culture. The number of metamyelocytes obtained from animals with liver fibrosis was < 63% after 48 h of cultivation; moreover, these cells were completely eliminated after 96 h of cultivation (Fig. 6B).

Stab and segmented neutrophils also showed the same behavior in culture (Fig. 7). The numbers of stab and segmented neutrophils derived from the bone marrow of control animals did not change significantly during 96 h of cultivation (Fig. 7). LMCC administration in animals with fibrosis increased the elimination rate of stab and segmented neutrophils in culture compared with that of neutrophils obtained from untreated animals with fibrosis (Fig. 7). The number of neutrophils obtained from animals with fibrosis almost linearly decreased during cultivation; moreover, these cells were completely eliminated after 96 h of cultivation (Fig. 7). LMCC administration in animals with fibrosis significantly decreased the number of stab and segmented neutrophils in the bone marrow. Moreover, the elimination rate of these cells during cultivation was higher than that of cells obtained from untreated animals with fibrosis (Fig. 7).

Therefore, in animals with fibrosis the distribution pattern of morphotypes of BMC changes. LMCC administration in animals with fibrosis did not significantly affect the behavior of BMCs or accelerate their elimination in primary culture compared with that of BMCs obtained from untreated animals with fibrosis.

## Discussion

The results of this study and of previous studies (Bozhkov et al., 2014; Bozhkov et al., 2015; Bozhkov et al., 2016) indicate the following:

1. Multiple sequential administration of copper (Cu) sulfate in experimental animals results in the accumulation of 60%–70% of injected copperions in the liver (Cichoż-Lach and Michalak, 2014). In liver cells, copperions bind to specific Cu binding proteins in the mitochondria and endoplasmic reticulum and induce oxidative stress (Kurguzova et al., 2015). This increases the amount of collagen in the liver to > 30%, which is a characteristic of fibrosis initiation (Ganai and Husain, 2017).

Data available on the quantitative and qualitative characteristics of the structural and functional response to of copperions suggest that oxidative stress is a key factor for fibrosis induction.

So, elimination of oxidative stress by activating antioxidant system, particularly by administration ofLMCC with antioxidant properties (Meena, 2013), may result in the restoration of the biochemical and physiologic characteristics of animals with Cu-induced liver fibrosis.

2. BMCs obtained from animals with Cu-induced liver fibrosis proliferated during the first 24 h of cultivation in primary culture. Thereafter, their number remained unchanged or decreased slowly. Moreover, the ratio of the main differentiated cell types in the bone marrow changed. The number of lymphocytes and stab neutrophils in the bone marrow of animals with Cu-induced liver fibrosis was lesscompared to control animals. The lifespan of stab and segmented neutrophils, myelocytes, and metamyelocytes also decreased in primary culture.

We believe that these epigenetic changes in neutrophils have an adaptive characteristic. After the suppression of inflammation, neutrophils are rapidly eliminated and new neutrophils with a new epigenotype corresponding to new exogenous stimuli are formed. LMCC administration

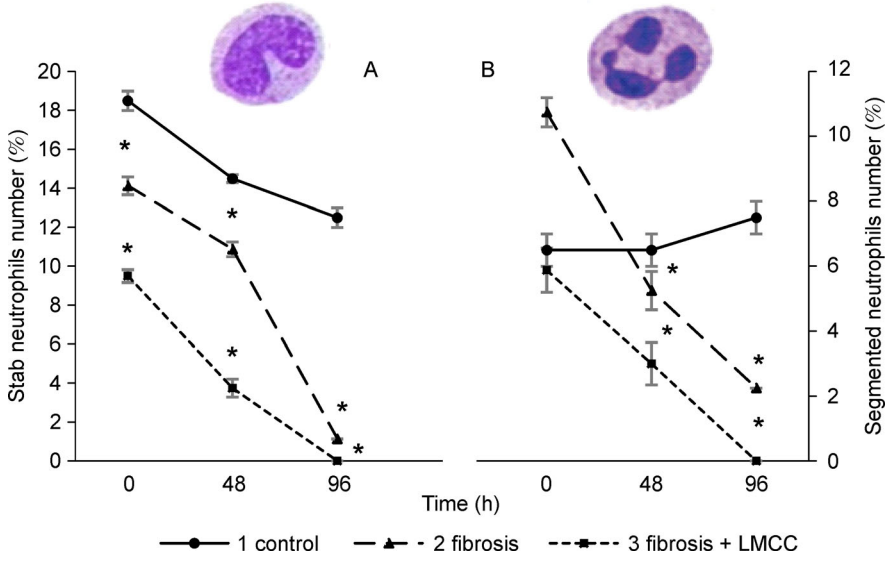


**Figure 7** Numbers of stab (A) and segmented (B) neutrophils obtained from the bone marrow of intact animals (1), animals with Cu-induced liver fibrosis (2), and animals with Cu-induced liver fibrosis treated with a triple dose of LMCC (0.1 g/100 g bodyweight *per os*) administered at 24-h intervals(3) during *in vitro* cultivation. * $p \leqslant 0.05$ compared with the control animals.

decreased the total number of different morphological cell types in the bone marrow of animals with Cu-induced liver fibrosis compared with that in the bone marrow of control animals and untreated animals with liver fibrosis. The distribution pattern of different cell morphotypes was similar to that in control animals, except for stab neutrophils.LMCC administration did not affect the lifespan of myelocytes and metamyelocytes but significantly reduced the lifespan of neutrophils in primary culture compared with that of cells obtained from untreated animals with liver fibrosis.

Moreover, LMCC administration imparted new properties to BMCs obtained from animals with Cu-induced fibrosis.

4. Administration of copper (Cu) sulfate to animals inhibited their growth and decreased their bodyweights and rectal temperature. Triple LMCC administration in animals with liver fibrosis normalized their growth rates and body temperature.

## Compliance with ethics guidelines

Anatoly Bozhkov, Eugeniy Ivanov, Yuliya Kuznetsova, Svetlana Ohiienko, and Anastasiya Bondar declare that they do not have any conflicts of interest related to this study. All animal experiments were performed in compliance with bioethical principles (Council Directive 86/609/EEC,1986) by considering the circadian rhythms of biological responses.

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